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Patentanmeldung Nr. **Patent application No.** **Demande de brevet n°**

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Immobilization of molecules on surfaces via polymer brushes

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In recent years there have been numerous approaches to fabricate sensor chips which are based on self-assembled monolayers (SAM's) of bifunctional molecules which couple sample molecules to be detected to the sensor surface. Typically, these molecules carry a silane or thiol/disulfide moiety in order to achieve a covalent bond with the inorganic surface and a functional group (e.g. amino or epoxide groups) which can be reacted with a test agent, often contained in biological samples in the form of an oligonucleotide, a protein or a polysaccharide etc.

In cases where the fast detection of oligonucleotides is required such layers can be brought in contact with solutions of synthetic test oligonucleotides and, subsequently, the hybridization of compatible strands from a test sample is detected, e.g. via fluorescence microscopy, if dye-labeled test nucleotides are used.

Although these techniques are well established for this purpose, the application of standard detection methods is problematic, especially in cases where the surface area available for the detection of one specific type of sample molecules is restricted, e.g. if a variety of molecules is to be analyzed in a parallel process, since the monolayers are limited in their graft density. As the number of hybridized double strands per surface unit of the sensor can not easily be increased, suitable detectors have to meet very high requirements with regard to their sensitivity, and the minimum surface area of a sensor necessary for the detection of one type of oligonucleotide can not be easily reduced. Moreover, the maximum graft density, i.e. one sample molecule per functional group of the couplers can

hardly be attained, since due to sterical hindrance on the two-dimensionally extended monolayer, only a fraction of the functional groups will be able to react with sample molecules. Thus, the overall graft density is low and normally unknown.

Various attempts have been made to overcome the problems outlined above. As regards the detection of oligonucleotides, it has been tried to increase the graft density on the surface by using oligomers or polymers which carry oligonucleotide strands (or functional groups for their attachment) together with suitable groups which allow the bonding of these oligomers or polymers to the surface of the sensor chip. Thus, a larger fraction of the bifunctional oligomer or polymer molecules which are coupled to the surface is able to immobilise test oligonucleotides in a sensor application.

However, the total oligonucleotide graft density is not significantly increased, because the graft density of the bifunctional oligomeric or polymeric molecules on the surface is limited. This is a consequence of the fact that the self-assembly of the oligomers or polymers is delayed for kinetic reasons, because once the sensor surface is covered with such molecules, further polymers will have to diffuse against a concentration gradient in order to reach the surface.

Accordingly, it is an object of the present invention to provide a sensor surface which is modified with a polymer monolayer comprising functional groups for the attachment of sample molecules, wherein the number of sample molecules which bind to a surface unit of the sensor is markedly increased compared to conventional monolayers of bifunctional molecules and wherein the density of the available coupling sites is higher than that obtained from the reaction of bifunctional polymers or oligomers with the

surface. In the specific case of the detection of oligonucleotides, the object can be expressed as the provision of a surface with a graft density of oligonucleotide strands which is higher than that created by coupling the respective oligonucleotides to a functionalised monolayer of low molecular weight compounds and also higher then that resulting from the reaction of polymers or oligomers comprising synthetic oligonucleotide single strands with the sensor surface.

This object has been achieved by a surface to which a multitude of copolymer chains is attached, which comprise units carrying one or more functional groups that allow a coupling reaction of the polymer with sample molecules. If, for example, such a polyfunctional polymer chain is allowed to couple with one or more synthetic oligonucleotides, corresponding nucleotide strands can subsequently be detected from a mixture of sample molecules after a hybridization reaction has taken place. Surprisingly it has been found that such an assembly of polyfunctional polymer chains, also referred to as a polymer brush, does not suffer from the problems of conventional detection methods where a high graft density could not be achieved. Moreover, since the flexibility of the polymer chains allows a complete coverage of the sensor surface, surface effects, e.g. during laser scanning, can be avoided.

The copolymer monolayer according to the present invention comprises a multitude of single copolymer chains which are attached to a surface. Preferably the bond between the polymer chain and the surface is covalent. Also it is preferred that the polymer chain is attached to the surface at one of it's terminals. The introduction of branched polymers is possible, if desired.

Furthermore, the copolymer is characterized in that it comprises at least one type of base polymer and at least one

type of polymer which carries the functional groups necessary to allow the coupling reaction of the polymer chains with the molecules to be detected. As a base polymer, a water swellable polymer is preferably chosen, i.e. a polymer comprising polar monomers which allow the interaction of the final polymer with water. Examples for suitable monomers which result in a water swellable polymer are acrylic acid or methacrylic acid and derivatives thereof, as esters or amides. A preferred monomer is e.g. dimethylacrylamide.

The monomers listed above can be used in admixture with other monomers known in the art

The ratio of base polymer monomers to those monomers which carry functional groups for the coupling reaction with the molecules to be detected is determined prior to the polymerization process in order to define the composition of the resulting polymer chain. Preferably, the comonomer ratio of the monomers forming the base polymer to the functionalized monomers ranges from 50/1 to 1/1.

The functional groups which are necessary to allow a coupling reaction of the polymer layer with the molecules to be detected are preferably present in the side chain of the polymer chains. Suitable functionalized monomers which are present in the copolymer of the polymer brushes are those monomers which comprise a polymerizable C-C double bond, as well as a further functional moiety that does not take part in the polymerization process. Preferably, this functional group is linked to the main polymer chain via a C_2-C_{10}, more preferably a C_3-C_7 alkyl chain as a spacer.

Preferred spacer molecules which carry these functional terminals and which, at the same time, allow their combination with polymerizable acrylic acids or methacrylic

acids are amino or hydroxy butanoic, pentanoic, hexanoic or heptanoic acids.

Suitable examples for monomers are acrylic acid or methacrylic acid esters or amides of C_2-C_{10} alcohols or amines which carry the necessary functional group at their other terminal. As a preferred functional monomer, the following compound can be employed for the present invention: N-methacryloyl-6-aminocapronic acid hydroxysuccinimide ester.

Functional groups which can be used for the purposes of the present invention should be chosen according to the molecules that are to be detected from a sample. Since the preferred application of the present invention is the detection of specific molecules in biological samples, the functional groups present within the polymer brushes will preferably bind to natural or synthetic biomolecules. Suitable functional moieties will preferably be able to react with nucleic acids, as DNA, RNA or PNA, polysaccharides, proteins including glycosidically modified proteins as antibodies, enzymes, cytokines, chemokines, peptidhormones or antibiotics, as well as oligonucleotides, peptides or aptamers.

Moreover, it should be possible to conduct the coupling reaction between the molecules to be detected or the synthetic oligonucleotides and the polymer chains under conditions which are not detrimental to the sample molecules, e.g. the reaction should be carried out in an aqueous solution.

Also, the coupling reaction should proceed at a reasonable rate so that the detection can preferably be accomplished within less than 24 hours without requiring extreme pH-values in the solution, i.e. in a pH range between 2 and 11.

Since most of the sample molecules, especially in biological or therapeutical applications, comprise sterically unhindered nucleophilic moieties, preferred coupling reactions with the polymer brushes comprise nucleophilic substitution or addition reactions. For example, synthetical test oligonucleotides are usually provided with a free amine group at one end (5′or 3′). Thus, exemplary functional groups provide, for example, a reactive double bond, an equivalent for a double bond (as e.g. an epoxy group) or a reactive leaving group. However, ionic or van-der-Waals forces can also be used to couple sample molecules to the polymer brushes if their functional groups are chosen accordingly.

With appropriate functional groups present in the polymer brushes, the polymer layers of the present invention can also be used in separation methods, e.g. as a stationary phase in chromatographic applications.

Suitable, preferred functional groups include active esters, e.g. N-hydroxysuccinimidyl esters or functional groups carrying an epoxide moiety, e.g. glycidyl ester groups.

The preferred method for the preparation of the polyfunctionalized polymer monolayers, which comprises a number of steps is described in the following:

In a first step, the surface is covered by a monolayer of polymerization initiators, which carry one or more functional groups suitable for their attachment to the surface. The groups which allow the initiation of the polymerization are usually chosen from peroxo groups or azo groups if a radical mechanism is to be used.

The functional groups for surface attachment have to be adapted to the sensor surface used. For the preparation of the initiator monolayer on metal oxides, especially silicon

oxide surfaces (evaporated or sputtered SiO_x layers, SiO_2 surfaces of silicon wafers, glass, quartz) chloro-silane moieties or alkoxysilanes are used. Thiol or disulfide groups can be employed for the modification of gold surfaces. However, the present invention is not restricted to inorganic surfaces. Polymer surfaces can also be used as substratres to carry the polymer monolayers, and there is also the possibility to include the starters for the polymerization reaction directly into such a surface forming polymer.

Upon initiation of the polymerisation reaction, preferably by a heating step or exposure to radiation in the presence of polymerizable monomers, polymer chains can be grown from the surface. If this technique is applied, graft densities of the resulting polymer monolayer can be achieved, that are inaccessible by other methods.

The polymerization can be carried out under standard reaction conditions known in the art.

Polymer layers prepared according to this method can be applied to a wide variety of surfaces, independent of their shape. Even surfaces which are inaccessible for conventional surface modification methods can be provided with the polymer monolayers according to the invention. Also, it is possible to create patterned arrays of the polymer monolayers by various means. One way are standard photolithographic processes that can either be applied after polymerization (photoablation of the polymers through masks) prior to this step (photodecomposition or photoablation of the initiator monolayer masks) or during the polymerization by means of photopolymerization through masks. Other possible techniques for the creation of patterned polymer monolayers are microcontact printing or related methods such as MIMIC, which may be applied during formation of the initiator layer or during polymerization. Finally, ink jet

techniques or other microplotting methods can be used to create patterned initiator monolayers which can subsequently be transferred to patterned polymer monolayers. Using any of these techniques, structures on the surfaces with dimensions in the micrometer range can be created.

For the detection of a successful coupling reaction of sample molecules with a polymer monolayer, a variety of techniques can be applied. In particular, it has been found that the polymer layers of the present invention undergo a significant increase in their thickness which can be detected with suitable methods.

If mixtures of oligonucleotides are to be analysed, synthetic oligonucleotide single strands can be reacted with the polymer monolayer.

Upon exposure to a mixture of oligonucleotides single strands, e.g. as obtained from PCR, which are dye-labled, only those surface areas which provide synthetic strands are complementary to the PCR product will show a detectable fluorescence signal upon scanning with a laser. In order to facilitate the parallel detection of different oligonucleotides sequences, printing techniques can be used which allow the separation of the sensor surface into areas where different types of synthetic oligonucleotide strands are presented to the test solution.

The present invention allows the fast analysis of different types of samples with an increased precision and /or reduced need of space in serial as well as parallel detection methods. The sensor surfaces according to the invention can therefore serve in diagnostical instruments for medical applications, e.g. for the detection of components in physiological fluids.

The present invention allows the provision of homogenically modified surfaces with superior graft density. By choosing the appropriate polymerization conditions, the chain length, and thus the thickness of the polymer layer can be controlled. Moreover, structured surfaces can be provided, e.g. by starting the polymerization from patterned arrays of initiator molecules. As a consequence, the polymer monolayers can be adjusted optimally to the respective applications.

Examples:

1. Formation of a polyfunctional polymer monolayer

A comonomer mixture of N,N-dimethyl-acrylamid (DMAA) and N-methacryloyl-6-aminocapronic acid hydroxy succinimid ester (C_6AE) is polymerised in dimethylformamide (DMF) as solvent. The monomer concentration is 4 mol/l at a molar ratio of the comonomers of DMAA/C_6AE=5/1. The polymerization is performed at 60°C. Prior to polymerization, the solutions are carefully degassed through at least 3 freeze-thaw-cycles in order to remove all oxygen traces. After polymerization, every sample is extracted with DMF for at least 10 hours.

2. Detection of oligonucleotides strands

The obtained surface is exposed to 1 nl of a 10 µM oligonucleotide-solution and the coupling reaction is allowed to proceed at 4o°C for two hours in a humid environment.

The synthetic oligonucleotide is 5-amino modified, and the solution is buffered with a 100 mM sodium phosphate buffer at a pH of 8.0. After the coupling reaction, the sensor surface is rinsed with the sodium phosphate buffer. In order to define the spatial extension of the specific types of

oligonucleotide on the sensor surface for parallel detection, the reactant was printed onto the polymer layer.

The surface thus prepared was allowed to react with a Cy5 labeled PCR product in a buffer of 2xSSC, 10% dextrane sulphate and 50% formamide for 12h at 28°C. The DNA content was 100 ng DNA /80 µl sample. After the hybridisation reaction has taken place, the surface was washed in SSC-buffer and the result was detected fluorometrically via laser activation with CCD camera. A fluorescence signal could only be detected for those areas which carried synthetic oligonucleotides complementary with the PCR product.

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Claims

1. Polyfunctional copolymer monolayer comprising an assembly of polymer chains attached to a surface, characterised in that the copolymer chains comprise units carrying one or more functional groups which allow a coupling reaction of the polymer with a sample molecule.

2. Copolymer monolayer according to claim 1, wherein the polymer chains are covalently attached to the surface.

3. Copolymer monolayer according to claim 1 or 2, wherein the functional groups are chosen from NHS ester groups or glycidylester groups.

4. Copolymer layer according to any of claims 1 to 3, wherein the sample molecule is chosen from proteins, polysaccharides, nucleic acids or fragments thereof.

5. Copolymer layer according to claims 1 to 4, wherein the sample molecule is comprised of a free functional group which allows a nucleophilic addition or a nucleophilic substitution reaction with the polymer chains of the monolayer.

6. Copolymer layer according to any of claims 1 to 5, wherein the copolymer comprises a water swellable base polymer.

7. Copolymer layer according to claim 6, wherein the base polymer is chosen from polyacrylates, polymethacrylates or polydimethylacetamines.

8. Surface carrying a polyfunctional copolymer monolayer according to any of claims 1 to 7.

9. Process for the production of a polyfunctional copolymer layer according to any of claims 1 to 7, comprising the steps of:

a) covering the surface with a monolayer of a polymerization initiator, which comprises one or more functional groups suitable for attachment to the surface.

b) initiating and carrying out a polymerization reaction in the presence of at least two types of monomers, wherein at least one type of monomer forms the base polymer and at least one type of a monomer carries functional groups which allow a coupling reaction of the obtained polymer chain with specific sample molecules.

10. Process according to claim 9 wherein the initiator molecules are covalently attached to the surface.

11. Process according to claim 10, wherein the initiator comprises a chloro-silane-, a disulphate- or a thiol group.

12. Process according to any of claims 9 to 10 wherein the initiator comprises an azo group.

13. Process for the detection of oligonucleotides single strands, comprising the subsequent steps of

a) providing a surface covered with a polyfunctional copolymer monolayer according to claim 8,
b) reacting the copolymer monolayer with synthetic oligonucleotides carrying suitable end groups,
c) exposing the surface to a solution of a test oligonucleotide or a mixture of test oligonucleotides which are dye-labled,

d) allowing a hybridization reaction to take place and subsequently removal of the non hybridized test oligonucleotides in a washing step

e) fluorometric detection of the hybridized test oligonucleotides.

14. Use of a surface according to claim 8 for the detection of a sample molecule.

15. Medical instrument, comprising a surface according to claim 8.